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# NETWORKED2-subfamily proteins regulate the cortical actin cytoskeleton of growing pollen tubes and polarised pollen tube growth

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## Summary

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**Key words:** actin, cytoskeleton, development, membrane, NET2A, pollen, signalling.

- We have recently characterised NET2A as a pollen-specific actin-binding protein that binds F-actin at the plasma membrane of growing pollen tubes. However, the role of NET2 proteins in pollen development and fertilisation have yet to be elucidated.
- To further characterise the role of Arabidopsis NET2 proteins in pollen development and fertilisation, we analysed the subcellular localisation of NET2A over the course of pollen grain development and investigated the role of the NET2 family using *net2* loss-of-function mutants.
- We observed NET2A to localise to the F-actin cytoskeleton in developing pollen grains as it underwent striking structural reorganisations at specific stages of development and during germination and pollen tube growth. Furthermore, *net2* loss-of-function mutants exhibited striking morphological defects in the early stages of pollen tube growth, arising from frequent changes to pollen tube growth trajectory. We observed defects in the cortical actin cytoskeleton and actin-driven subcellular processes in *net2* mutant pollen tubes.
- We demonstrate that NET2 proteins are essential for normal actin-driven pollen development highlighting an important role for the NET2 family members in regulating pollen tube growth during fertilisation.

## Introduction

The male reproductive cycle of angiosperms is an elaborate process, comprising the development and generation of highly specialised pollen grain cells within the anther locule; a process known as microgametogenesis. During fertilisation, the mature pollen germinates upon the stigma and extends a growing pollen tube, carrying nonmotile sperm cells through the pistil to the ovule, guided by extracellular signals secreted by the pistil (Kanaoka & Higashiyama, 2015).

The actin cytoskeleton is crucial for pollen development, germination and pollen tube growth (Gervais *et al.*, 1994; Zonia *et al.*, 1999; Gibbon *et al.*, 1999; Vidali *et al.*, 2001). During microgametogenesis actin regulates some processes including nuclear migration, mitotic divisions and cell expansion. Actin is also implicated in regulating specific reorganisations of cellular structure that occur in developing pollen, including fragmentation and dispersal of the pollen vacuole, and structuring of the plasma membrane, both of which are dependent on clathrin-mediated endocytosis (CME; Kang *et al.*, 2003; Yamamoto *et al.*, 2003; Kim *et al.*, 2013). In pollen germination and tube growth, actin regulates targeting of Golgi-derived secretory vesicles to the growing tip, cytoplasmic streaming and endocytosis (Vidali &

Helper, 2000; Lee *et al.*, 2008; Moscatelli *et al.*, 2012; Rounds *et al.*, 2014; Volger *et al.*, 2015). During fertilisation, the actin cytoskeleton is believed to be controlled in response to extracellular signals secreted by the pistil to regulate pollen tube directional growth and navigation to the ovule. Each of these processes is mediated by highly organised and dedicated actin arrays, which are in turn regulated by a large number of actin-binding proteins.

We have recently identified one such protein, NETWORKED 2A (NET2A), a pollen-specific member of the NET superfamily of actin-binding proteins. We demonstrated that NET2A bound F-actin to the plasma membrane through interaction with the membrane-integral receptors, POLLEN RECEPTOR-LIKE KINASES 4 and 5 (PRK4 and PRK5), to form physical links between the actin cytoskeleton and plasma membrane, so-called 'actin-membrane contact sites' (AMCSs; Duckney *et al.*, 2017). PRKs are upstream regulators of extracellular signal transduction to the actin cytoskeleton during fertilisation (Tang *et al.*, 2002, 2004; Lee *et al.*, 2008; Wengier *et al.*, 2010; Huang *et al.*, 2014; Takeuchi and Higashiyama, 2016), suggesting important roles for NET2 subfamily proteins as downstream regulators of actin in response to extracellular guidance cues during fertilisation (Duckney *et al.*, 2017). However, the importance of NET2 proteins in pollen has yet to be identified.

Here, we further investigated the potential roles for NET2 subfamily proteins in the male gametophyte using multiple approaches. Firstly, we analysed the subcellular localisation of NET2A to the actin cytoskeleton during pollen development, germination and pollen tube growth. We noted a dynamic association of NET2A with the actin cytoskeleton as it underwent structural reorganisations at specific stages in pollen development and tube growth, suggestive of its involvement in the regulation of actin in the male gametophyte. We next confirmed an important role for NET2 proteins in pollen tube growth through reverse genetic analysis of specific combinations of *net2* mutants. We demonstrate that NET2 proteins are important in organising F-actin at the plasma membrane and regulating actin-driven subcellular processes including vesicle trafficking and cytoplasmic streaming in pollen tubes, potentially through the regulation of previously characterised AMCSs (Duckney *et al.*, 2017).

Our data establish an important role for NET2 proteins in the male gametophyte and in angiosperm fertilisation. We further demonstrate the importance of the previously characterised NET2-mediated connections between the actin cytoskeleton and the plasma membrane in the organisation of the cytoskeleton in pollen tube growth.

## Materials and Methods

### Plant material and transformations

*Arabidopsis thaliana* (L.) Heynh. (Col-0 ecotype) was used to generate stable Arabidopsis transformants using the floral dipping method according to Zhang *et al.* (2006). *Arabidopsis thaliana* Col-0 lines stably transformed with *pNET2A:NET2A-GFP* alone, or *pNET2A:NET2A-GFP* and *pLAT52:FABD2-RFP* together were generated previously (Deeks *et al.*, 2012; Duckney *et al.*, 2017). Seeds were grown on half-strength Murashige & Skoog (½MS) agar or compost in a growth chamber with a 16 h : 8 h, day : night cycle, with 22°C : 18°C, day : night temperature.

### Molecular biology

To generate the *pLat52:GFP-RabA4B* construct, the cDNA of RabA4B was cloned from Arabidopsis Col-0 pollen using PCR and cloned into the binary vector pB7GWF52 using the Gateway cloning system (Invitrogen).

For the *pLat52:Lifeact-mNeonGreen* construct, a codon-optimised coding sequence of Lifeact-mNeonGreen was synthesised (IDT) cloned into pB7GW52 using the Gateway cloning system (Invitrogen).

For pollen-specific expression of constructs under the *pLAT52* promoter, the binary expression vectors pB7GW52 (for expression with no tag) and pB7GWF52 (for expression of N-terminal GFP) were generated from pB7GW2 and pB7GWF2 respectively, as previously described (Duckney *et al.*, 2017). The *pLAT52* promoter sequence (Twell *et al.*, 1990) was PCR amplified with added 5' *SacI* and 3' *SpeI* sites. Excision of the *CaMV* 35S:promoter sequence was performed using *SacI/SpeI* double

restriction enzyme digestion, and the 5'-*SacI*-*pLAT52*-*SpeI*-3' DNA fragment was ligated into the excision site using T7 DNA ligase (NEB).

### Reverse-genetics

The *net2a.1* (GABI\_234H12), *net2b.1* (GABI\_486H12), *net2b.2* (GABI\_132E08), *net2d.1* (SALK\_042729) and *net2d.2* (SALK\_035401) T-DNA insertion mutants were ordered from NASC. Homozygosity was confirmed using the primers described in Supporting Information Table S1. Total RNA was extracted from inflorescences (Qiagen) and cDNA was synthesised using Superscript III (Invitrogen). RT-PCR was used to confirm the absence of full-length transcripts in the *net2* T-DNA mutants using the primers listed in Table S1. RT-PCR amplification of the housekeeping gene EF1 $\alpha$  was performed as a control, and equal amounts of Col-0 and mutant cDNA were used.

For CRISPR/Cas9-mediated generation of *net2c* mutants, two *NET2C* gRNA spacer sequences were chosen as Cas9 targets: target 1 (sense 5'-3': TTTTGTTAGAAGAATCTTTCA) and target 2 (sense 5'-3': GAATGGACTTGCAAAGTATT) on the + strand. Target specificities were evaluated with CAS-OFFINDER, using an algorithm for potential off-target sites of Cas9 RNA-guided endonucleases (<http://www.rgenome.net/cas-offinder>; Bae *et al.*, 2014). The two *NET2C* gRNA expression cassettes were constructed by PCR using pCBC-DT1T2 as a template according to Xing *et al.* (2014). The *NET2C-DT1DT2* gRNA expression module was cloned using a Golden Gate cloning protocol into the vector pHEE401E allowing egg cell-specific EC1.2en:EC1.1 promoter-controlled expression of 3 $\times$  FLAG-NLS-zCas9-NLS and conferring hygromycin resistance in transformed plants (Wang *et al.*, 2015). Transformants were genotyped and sequenced using the primers listed in Table S1.

### Live cell imaging

Live pollen grains and germinated pollen tubes were imaged using laser scanning confocal microscopy (LSCM) (Leica TCS SP5 microscope). Whole anthers were excised and dissected to permit visualisation of developing grains through the anther wall. Images were acquired in multitrack sequential mode with laser line switching when imaging co-localisation of multiple fluorophores. Rapid differential interference contrast (DIC) imaging was performed using the Applied Precision OMX BLAZE Super-Resolution microscope (GE Healthcare) in DIC operating mode at a rate of 286 MHz.

Image deconvolution was performed using Huygens deconvolution professional suite software (Scientific Volume Imaging).

### 4',6-Diamidino-2-phenylindole (DAPI) staining of developing pollen grains

DAPI staining of pollen grains was performed as described by Park *et al.* (1998). Whole anthers were dissected and mounted on a microscope slide in 10  $\mu$ l DAPI staining solution (0.1 M phosphate buffer, pH 7 (57.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 42.2 mM NaH<sub>2</sub>PO<sub>4</sub>),

1 mM EDTA, 0.1% Triton X-100,  $0.4 \mu\text{g ml}^{-1}$  DAPI). The anthers were gently squashed under a coverslip to release the pollen grains into the DAPI staining solution. The samples were incubated for 10–30 min before imaging using LSCM.

### Pollen tube growth assays

*In vitro* germination of Arabidopsis pollen was performed on solid medium as previously described (Li *et al.*, 1999). Germination medium consisted of 18% (w/v) sucrose, 0.01% (w/v)  $\text{H}_3\text{BO}_4$ , 1 mM  $\text{Ca}(\text{NO}_3)_2$ , 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$  and 0.5% (w/v) Agarose Type VII-A (Sigma), pH 7. Mature pollen from fully opened flowers was dusted onto solid germination medium and samples were incubated in a dark humid environment at  $22^\circ\text{C}$  for >4 h. Subsequently, germinated pollen was analysed using LSCM as described above. Mitotracker Red (Invitrogen) was used for visualisation of mitochondria in growing pollen tubes.

Semi *in vivo* pollen germination was performed as previously described (Palanivelu & Preuss, 2006).

Aniline blue staining of fertilised pistils was performed as previously described (Deeks *et al.*, 2007). Emasculated mature Col-0 pistils were fertilised with Col-0 and *net2a/net2b/net2c/net2d* mutant pollen grains and excised after either 6 h or 24 h after pollination before staining and imaging.

## Results

### Dynamic rearrangement of NET2A subcellular localisation patterns during pollen grain development and germination

The pollen-specific NETWORKED protein, NET2A, has recently been implicated in mediating AMCSs in growing pollen tubes (Duckney *et al.*, 2017). In this study, we investigated whether NET2A may also play a role in pollen development and germination. NET2A subcellular localisation was investigated in live Arabidopsis pollen grains stably expressing native promoter-driven NET2A-GFP. NET2A-GFP was noted to exhibit distinct localisation patterns at specific stages of microgametogenesis, undergoing dynamic reorganisations throughout the pollen developmental programme. To observe successive changes in NET2A-GFP subcellular localisation over the course of pollen grain development, serial analysis was performed on pollen grains taken from each bud on an individual inflorescence. As previously described (Regan & Moffatt, 1990; Sanders *et al.*, 1998), the sequential position of each bud on the inflorescence represents a unique developmental stage of each bud at that point in time and, therefore, the specific developmental stage of the pollen within. DAPI staining was performed to identify the developmental stage at which the specific NET2A arrays occurred.

During microgametogenesis, NET2A-GFP was first observable at discreet, static punctae within developing bicellular grains (Fig. 1a-i). These NET2A-GFP punctae did not appear to be specifically associated with the pollen plasma membrane, as described previously in pollen tubes (Deeks *et al.*, 2012; Duckney *et al.*, 2017), but alternatively distributed throughout the cytoplasm. In older flowers with further developed bicellular pollen,

NET2A-GFP subsequently localised to a notably distinct pattern, decorating highly organised, transversely orientated cables at the cell cortex, and a finer meshwork of filaments extending throughout the cytoplasm (Fig. 1a-ii). This NET2A-GFP subcellular localisation pattern was designated as the 'NET2A transverse array'. The transverse array displayed marked polarity within the bicellular grains; with the cortical actin cables consistently aligning transversely to the cell longitudinal axis and the exine aperture (Fig. 1b). In late-bicellular and early-tricellular pollen, NET2A-GFP decorated a highly filamentous network at the cell cortex, which extended throughout the cytoplasm, connecting to a basket structure surrounding the vegetative cell nucleus (VCN) (Fig. 1a-iii). This NET2A-GFP-decorated network appeared to be static under observation using time-lapse LSCM. We termed this NET2A-GFP localisation pattern the 'NET2A filamentous array'. Subsequently, before dehiscence, NET2A-GFP was observed to localise to a dense network of fine filaments at the cell cortex, interconnected with a sparser filamentous network throughout the cytoplasm. This localisation pattern was named the 'NET2A cortical array' (Fig. 1a-iv). Finally, in mature dehiscent pollen grains NET2A-GFP localisation was fully cytosolic and did not decorate filaments or punctae. Dehiscence occurs over a period of 6 h (Smyth *et al.*, 1990) therefore narrowing the change in NET2A localisation to this timeframe.

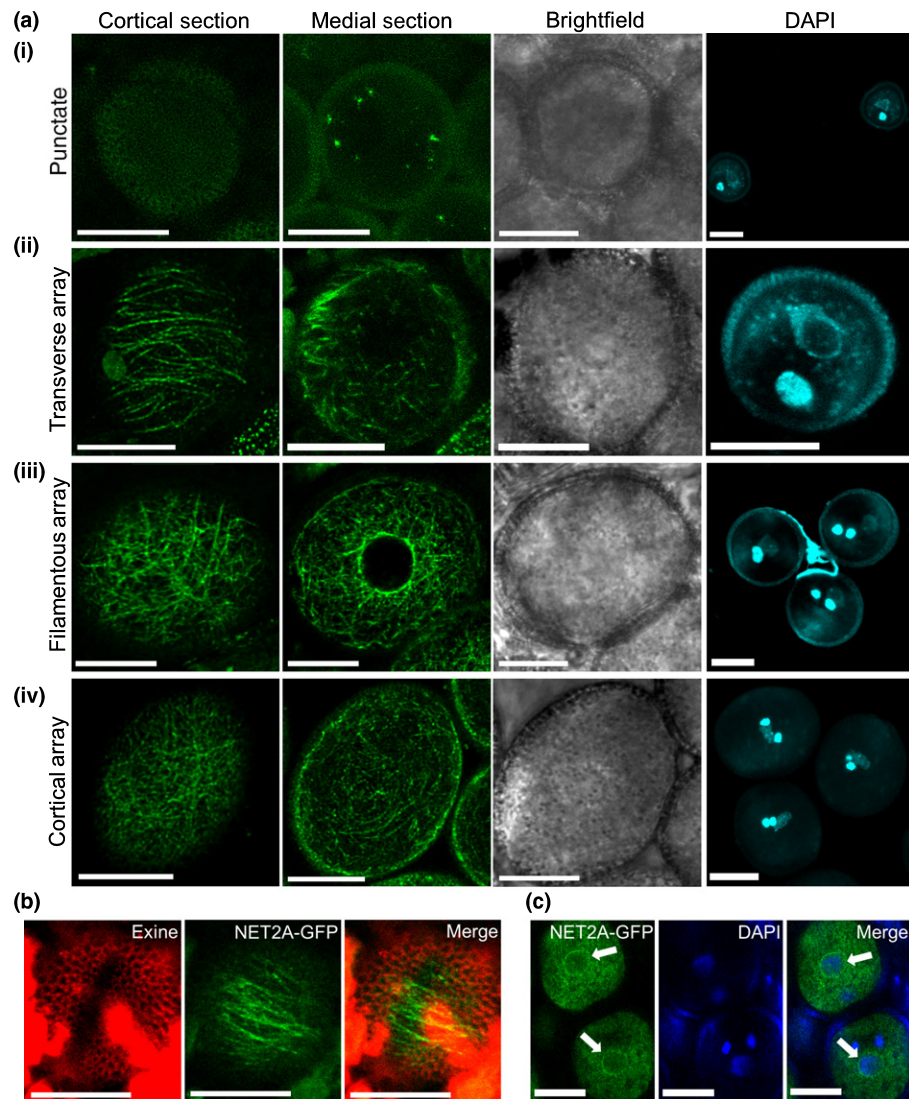
Therefore, we demonstrate that NET2A exhibits distinctive localisation patterns at different stages of pollen development. The NET2A-GFP-decorated punctae and filamentous systems were static under observation for periods of 1 min and it is, therefore, likely that the highly distinct NET2A-GFP arrays observed are a result of extensive reorganisations at specific stages of pollen development.

### NET2A localises to F-actin during microgametogenesis

NET2A is implicated in the regulation of actin in growing pollen tubes (Duckney *et al.*, 2017) and it is now possible that NET2A may also play a role in the regulation of actin-dependent processes in developing pollen. Therefore, we investigated whether NET2A may localise to F-actin in the developing grains. Co-localisation of NET2A and F-actin was visualised in live pollen grains of stable Arabidopsis transgenic lines co-expressing native promoter-driven NET2A-GFP and the actin marker construct, FABD2-RFP, expressed under the pollen-specific promoter, *pLAT52* (Ketelaar *et al.*, 2004; Duckney *et al.*, 2017). Here, NET2A-GFP co-localised with F-actin in each of the NET2A arrays described above. Furthermore, we observed F-actin to undergo a series of developmentally coordinated rearrangements in tandem with NET2A.

Expression of *pLAT52* promoter-driven FABD2-RFP was first observable in bicellular pollen grains exhibiting the NET2A transverse array. F-actin localised to highly organised cables at the cell cortex, aligned perpendicular to the cell longitudinal axis, forming an 'actin girdle' structure (Fig. 2a). NET2A-GFP co-localised with the actin girdle, suggestive that NET2A may decorate the actin cytoskeleton in the transverse array. In late-bicellular and early-tricellular pollen grains, striking co-localisation between NET2A-GFP and F-actin was evident in the





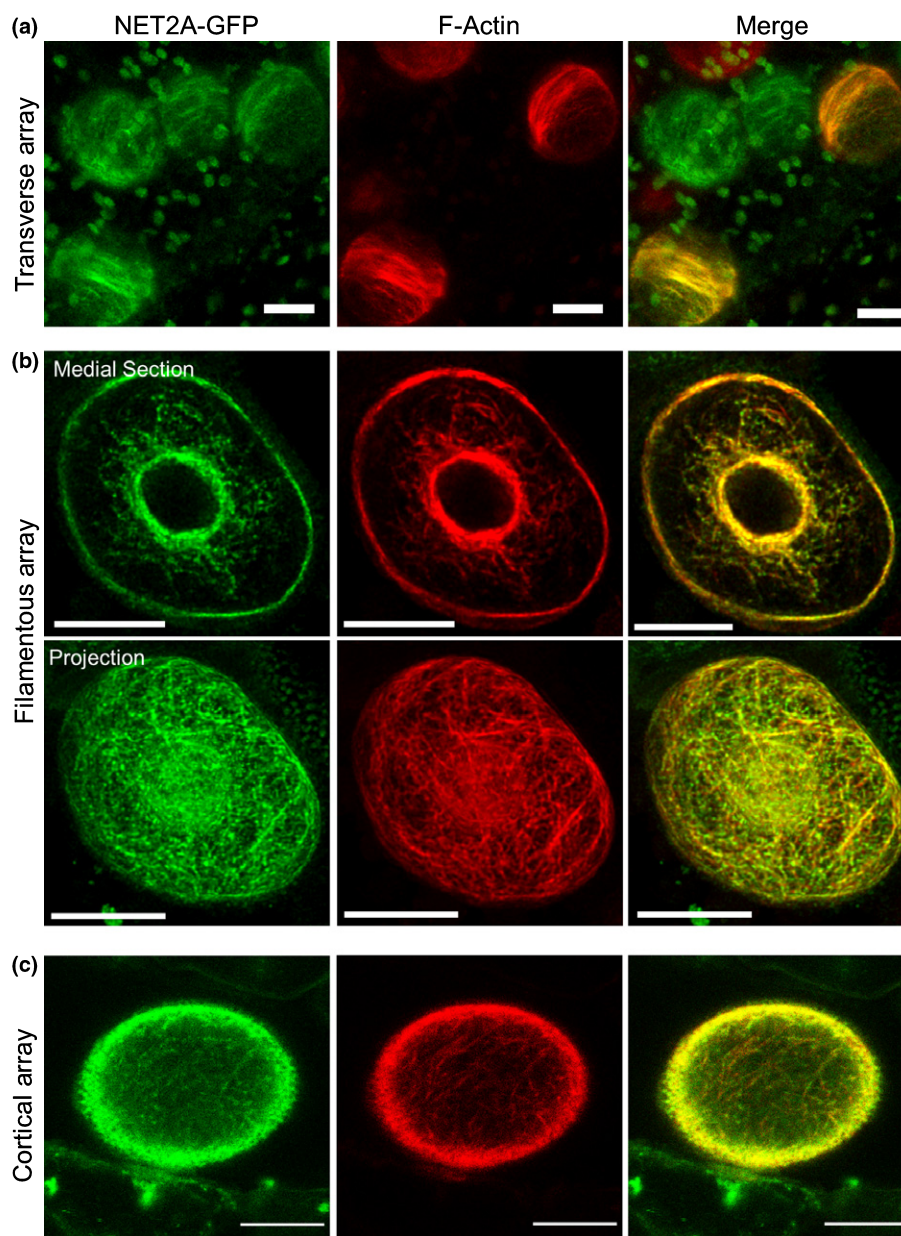
**Fig. 1** NET2A undergoes dynamic rearrangements in subcellular localisation during *Arabidopsis* pollen grain development. (a) NET2A-GFP subcellular localisation observed at progressive stages of grain maturation. The subcellular localisation of NET2A-GFP at each developmental stage at the cell cortex and cell midplane, the corresponding brightfield image and pollen grains taken from the same bud stained with DAPI are depicted. (i) NET2A-GFP expression was first observed as discrete, static, cytoplasmic punctae. (ii) NET2A transverse array. NET2A localises to transversely orientated cortical cables at the equatorial region of the cell. (iii) NET2A filamentous array. NET2A localises to continuous filamentous networks at the cell cortex, cytoplasm and surrounding the vegetative cell nucleus (VCN). (iv) NET2A cortical array. NET2A localises to a dense meshwork of filaments at the cell cortex, continuous with a sparse network of fine cytoplasmic filaments. (b) In the NET2A transverse array, cortical NET2A-GFP cables lie transversely to the pollen grain longitudinal axis and the exine groove. (c) NET2A encircles the VCN in the NET2A filamentous array (as indicated by the white arrows). Bars, 10  $\mu$ m.

filamentous array (Fig. 2b). F-actin localised to a cortical filamentous network, interconnected with cytosolic filaments, continuous with an actin basket surrounding the VCN. NET2A-GFP co-localised with F-actin at the VCN nuclear basket, cytosolic filaments and cortical actin networks. Finally, NET2A also localised to the actin cytoskeleton in the NET2A cortical array (Fig. 2c). A dense network of fine actin filaments was present at the cell cortex, continuous with a sparse network of fine actin filaments in the cytoplasm. NET2A-GFP co-localised with F-actin at both the cell cortex and in the cytoplasm.

Therefore, the actin cytoskeleton reorganises successively into highly distinctive structures at specific stages of *Arabidopsis* pollen grain development, to which NET2A localises.

### Changes in NET2A distribution occur during pollen germination

The actin cytoskeleton has a fundamental role in pollen germination (Gibbon *et al.*, 1999), during which it undergoes highly dynamic rearrangements in structure (Volger *et al.*, 2015). Similarly, NET2A localises to drastically different patterns before and after pollen germination. Whilst NET2A is cytosolic in mature pollen grains before germination (Fig. 3a), it localises to focal AMCSs at the shank plasma membrane of growing pollen tubes (Deeks *et al.*, 2012; Duckney *et al.*, 2017). We therefore investigated how NET2A localisation patterns are redistributed during pollen germination, as the actin cytoskeleton undergoes dynamic



**Fig. 2** NET2A localises to actin filaments during *Arabidopsis* pollen grain development. NET2A-decorated filaments co-localised with F-actin, labelled with the genetically encoded actin marker, FABD2-RFP, in each NET2A array. NET2A-GFP co-localised with FABD2-RFP during the NET2A transverse array (a), the NET2A filamentous array (b) and the NET2A cortical array (c). Bars, 10  $\mu$ m.

changes in structure. Fig. 3a shows the cytosolic localisation of NET2A-GFP in mature pollen before germination. Here, NET2A-GFP did not localise to filamentous networks or punctae. In pollen grains undergoing germination, NET2A-GFP was largely cytosolic, however discrete NET2A-GFP foci had formed at the cell cortex in the vicinity of the bulge site, where the nascent pollen tube germinates (Fig. 3b). In newly germinated pollen tubes, NET2A-GFP localised to punctae at the plasma membrane of the base and shank of the growing tube, similar to that found in fully germinated pollen tubes as previously described (Fig. 3c; Duckney *et al.*, 2017). Taken together, it

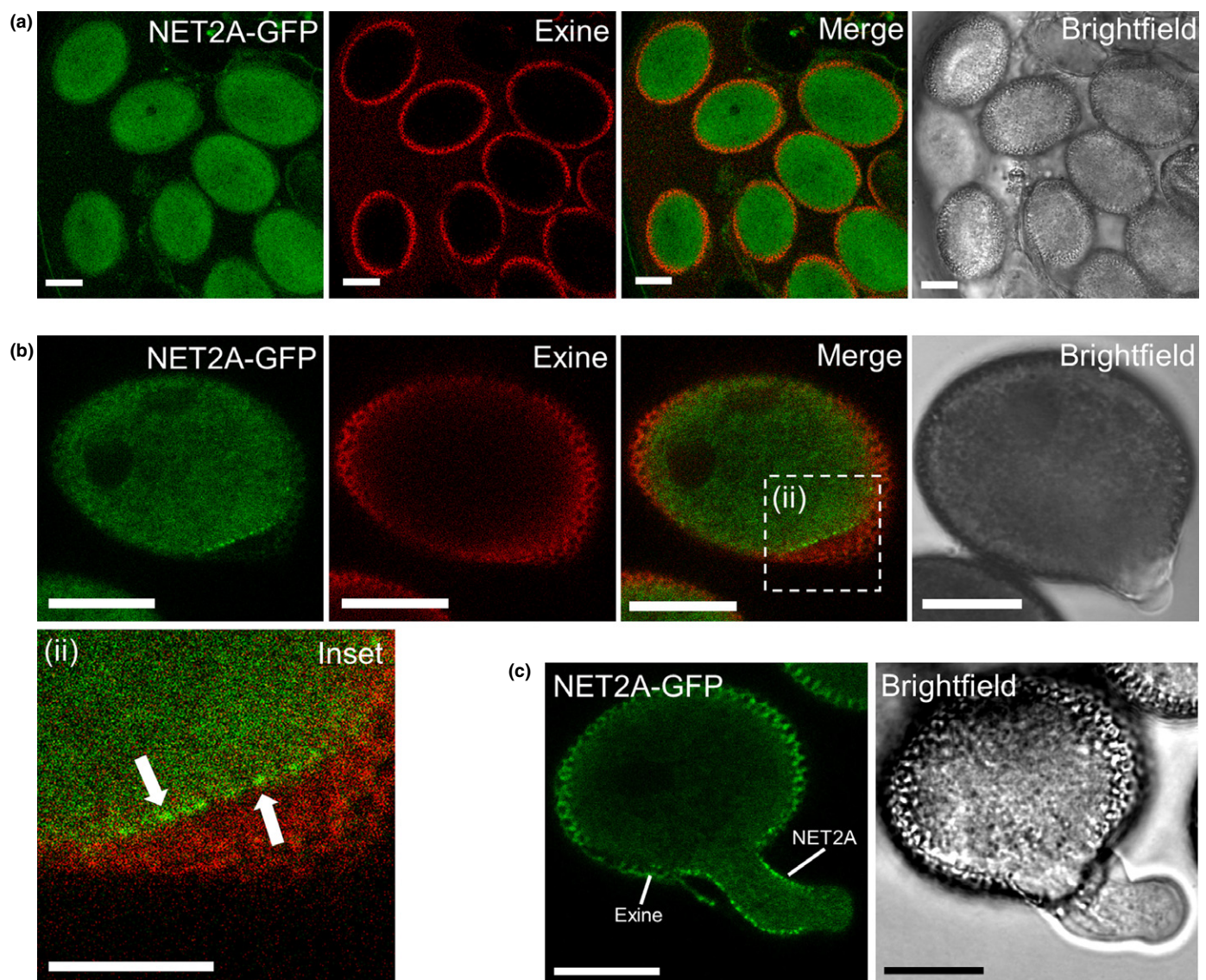
appears that NET2A is recruited from the cytosol to punctae at the site of germination and, during pollen tube growth, is deployed at the plasma membrane of the newly established shank region as the maturing membrane becomes increasingly distal from the growing tip (Fig. 3d). NET2A punctae are sites of actin-membrane interactions in growing pollen tubes (Duckney *et al.*, 2017). Further to this, these NET2A punctae are also present at the pollen grain germination site before and after germination. Therefore, we propose that NET2A may also mediate actin-membrane interactions at discrete foci at the site of pollen tube outgrowth during germination.



### *net2* loss-of-function mutants exhibit defects in pollen tube growth

We have shown that NET2A localises to F-actin in developing pollen and establishes actin-membrane contacts sites at the PM of germinating and growing tubes (Duckney *et al.*, 2017). We therefore hypothesised a function for NET2 proteins in the regulation of actin during pollen development and tube growth. To investigate the role of NET2 in pollen, we generated loss-of-function mutants of each member of the NET2 protein family. We obtained T-DNA insertion mutants for *NET2A*, *NET2B* and *NET2D*, (Fig. 4a,b) and used CRISPR/Cas9 technology to generate loss-of-function mutant alleles of *NET2C* (Fig. 4c,d). Double, triple and quadruple mutants of each combination of

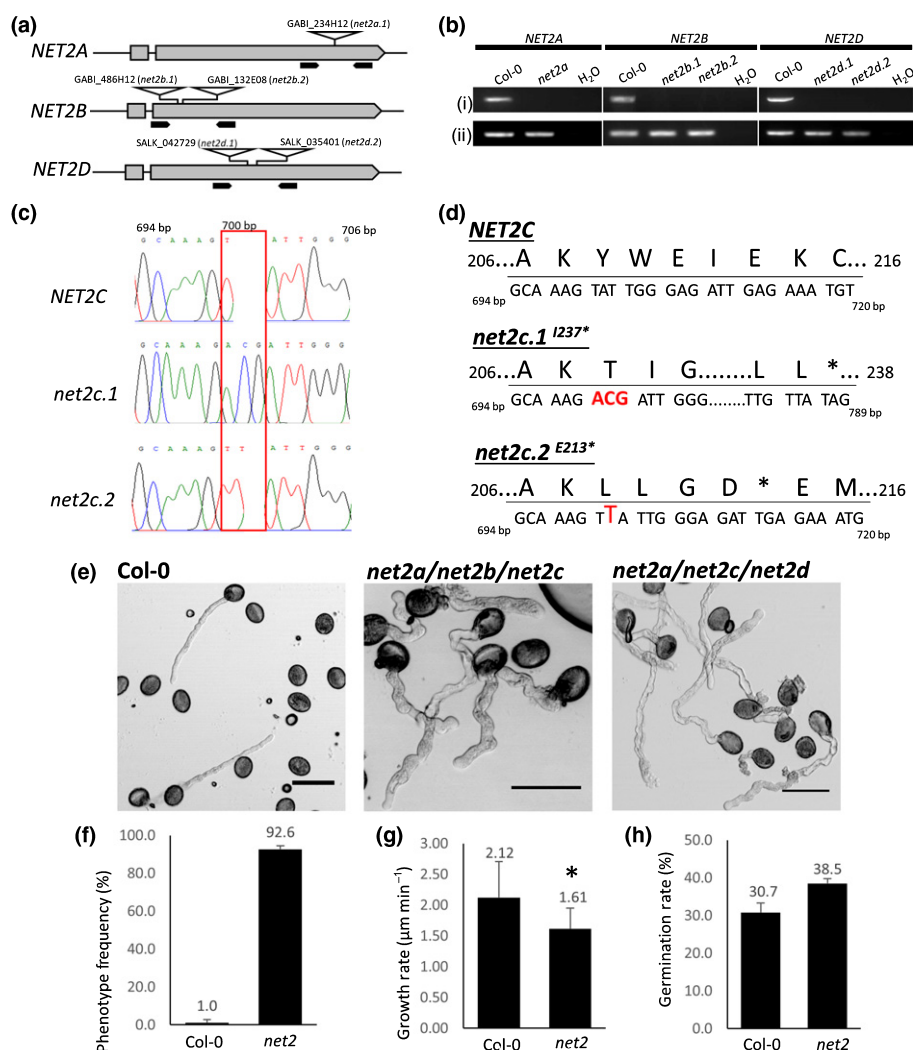
*net2* mutant alleles were generated and analysed for defects in pollen development and tube growth (Fig. S1). We observed phenotypic defects in specific triple mutant combinations and defective pollen tube morphology was evident in *net2a/net2b/net2c* and *net2a/net2c/net2d* mutants, which exhibited a 'zig-zagging' profile (Fig. 4e). We also noted that these mutant pollen tubes exhibited periodic depolarisation along the length of the tube, consistent with tip growing cells of mutants of other actin regulatory proteins such as CAP1 (Deeks *et al.*, 2007). *net2a/net2b/net2c/net2d* quadruple mutants appeared to exhibit no increased severity in pollen tube growth defects (Fig. S1). No phenotype was observed in single or double *net2* mutant combinations. The data demonstrate important roles for the NET2 protein subfamily in pollen.



**Fig. 3** NET2A undergoes dynamic reorganisation during Arabidopsis pollen germination. (a) NET2A localises to the cytosol in mature dehiscence pollen grains. (b) During germination, NET2A localises to punctae at the cell cortex, specifically facing the site of germination (as indicated by the white arrows). (c) NET2A localises to punctae at the shank plasma membrane of newly germinated pollen tubes, similar to as described in growing pollen tubes (Deeks *et al.*, 2012; Duckney *et al.*, 2017). NET2A-GFP punctae in the pollen tube shank (indicated as NET2A) are distinguishable from exine autofluorescence (labelled as 'exine'). Bars, 10 µm, with an exception for (b ii), for which bar, 5 µm.

Pollen tube growth is restricted to the cell tip, indicating that defects in pollen tube morphology are likely to arise from defective tip growth. We therefore used time-lapse microscopy to study the progression of tip growth in *net2a/net2b/net2c* mutant pollen over time. Whereas Col-0 pollen tubes largely extend in a uniform direction, the pollen tubes of *net2a/net2b/net2c* mutants appear unable to maintain a constant growth trajectory (Video S1), resulting in the observed morphological defects. The percentage of *net2a/net2b/net2c* pollen tubes exhibiting this morphological defect was observed to be 92.6%, compared with 1.0% in Col-0 pollen (Fig. 4f). We also noted a slight, but statistically

significant, decrease in the growth rate of *net2a/net2b/net2c* mutant pollen tubes compared with that of Col-0 pollen tubes (Fig. 4g;  $P < 0.05$ ). Interestingly, a more pronounced reduction in pollen tube growth rate was observed during pollen tube growth through the stigma in semi-*in vivo* growth assays and *in vivo* during fertilisation (Figs S2, S3). We could observe no reduction in pollen tube germination in *net2a/net2b/net2c* mutant pollen (Fig. 4h), leading us to speculate that the *net2* loss-of-function phenotype had not become apparent until after pollen germination. We concluded that the NET2 protein subfamily has important roles in the regulation of pollen tube growth.



**Fig. 4** Loss of function of Arabidopsis NET2 proteins results in defective pollen tube morphology. (a) Diagrammatic representation of T-DNA mutant alleles of *NET2A*, *NET2B* and *NET2D* used in this study. Gene exons are represented by grey boxes. The locations of the T-DNA insertion are depicted by white triangles. The positions of primers used for RT-PCR analysis are depicted by black arrows. (b) *NET2A*, *NET2B*, *NET2D* T-DNA alleles used result in disrupted gene transcription. (i) Gene-specific RT-PCRs: the locations of the primers used are depicted in Fig. 4a. (ii) Control RT-PCR of EF-1a housekeeping gene. (c) *net2c* CRISPR mutant alleles used in this study. The red box indicates the regions of the *NET2C* gene modified by the Cas9 enzyme. (d) Effects of *net2c.1* and *net2c.2* CRISPR mutations on the *NET2C* open reading frame. *net2c.1* results in I237\* and *net2c.2* results in E213\*. (e) *net2a/net2b/net2c* and *net2a/net2c/net2d* triple mutants exhibit morphological defects. Bars, 50  $\mu\text{m}$ . (f) Frequency of defective pollen tube growth in Col-0 and *net2a/net2b/net2c* mutants. 187 Col-0 and 268 mutant pollen tubes were analysed. (g) Pollen tube growth rate ( $\mu\text{m min}^{-1}$ ) is slightly reduced in *net2a/net2b/net2c* mutants. 14 Col-0 and 14 mutant pollen tubes were analysed. The asterisk indicates statistical significance ( $P < 0.05$ ; calculated using Student's *t*-test). (h) The pollen germination rate of *net2a/net2b/net2c* mutants is similar to that of Col-0. 919 Col-0 and 731 mutant pollen grains were analysed. Chart error bars represent standard deviation of the mean.



## The cortical actin cytoskeleton is disorganised in *net2* loss-of-function mutants

We have demonstrated previously that NET2 proteins bind cortical longitudinal actin cables to the plasma membrane in growing pollen tubes (Duckney *et al.*, 2017), suggesting an important role in actin-driven pollen tube growth. To investigate a role for NET2 proteins in the regulation of actin in growing pollen tubes, we investigated the actin cytoskeleton of *net2a/net2b/net2c/net2d* mutant pollen to identify potential defects in the pollen tube cytoskeleton.

To visualise the actin cytoskeleton in growing pollen tubes, we expressed the actin marker, Lifeact (Smertenko *et al.*, 2010) linked to the 5'-end of the fluorescent protein, mNeonGreen, under the *pLAT52* promoter. The actin cytoskeleton of the *net2* quadruple mutant pollen tubes was observed to be disrupted, (however no defects in actin organisation were observed in developing *net2a/net2b/net2c/net2d* mutant pollen grains expressing Lifeact-mNeonGreen; Fig. S4). Using CLSM we revealed that Col-0 pollen tubes displayed thick, longitudinally aligned actin cables extending over much of the length of the growing pollen tube. By contrast, *net2* quadruple mutant pollen tubes exhibited a sparse array of short and fine actin filaments (Fig. 5a). To accurately compare the structure of the actin cytoskeleton of Col-0 and *net2* quadruple mutant pollen tubes, we used rapid DIC imaging to capture the 3D structures of the pollen tube cytoskeleton in high temporal resolution (Fig. 5b). The data demonstrate that the actin cytoskeleton was disorganised in the *net2* quadruple mutant tubes, appearing fragmented and frequently misaligned to the longitudinal axis of the growing pollen tube. Furthermore, the prominent longitudinal actin cables of the Col-0 pollen tubes were markedly absent from the *net2* quadruple mutants.

As NET2 proteins are implicated in the regulation of AMCSs at the pollen tube plasma membrane, we next investigated potential roles for NET2 in the organisation of F-actin at the cell cortex. Analysis of the actin cytoskeleton at the cell cortex of *net2* quadruple mutant pollen tubes revealed a decrease in cortical F-actin compared with Col-0 pollen tubes (Fig. 5c-i). We recorded a density of 0.97 actin filaments  $\mu\text{m}^{-1}$  at the cell cortex of Col-0 pollen tubes, whereas the density of cortical F-actin in *net2* quadruple mutants was significantly lower, at 0.57 actin filaments  $\mu\text{m}^{-1}$ . Furthermore, *net2* quadruple mutants also exhibited a reduced proportion of cortical actin filaments (within 1  $\mu\text{m}$  of the cell cortex) relative to core actin filaments ( $>1 \mu\text{m}$  of the cell cortex). Orthogonal cross-sections of 3D confocal images of growing pollen tubes expressing Lifeact-mNeonGreen revealed the positions of longitudinal actin cables in the cell (Fig. 5d-i, d-ii). We observed a cortical F-actin ratio of 2.1 in Col-0 pollen tubes and a significantly reduced cortical F-actin ratio of 0.6 in *net2* quadruple mutant pollen tubes. Taken together, our data indicate that *net2* quadruple mutants exhibited a reduction in cortical F-actin at the pollen tube plasma membrane. This observation was consistent with the hypothesis that NET2 proteins may serve as linkers or anchors of F-actin at the plasma membrane and demonstrated an important function for the NET2

protein subfamily in the regulation of the pollen tube actin cytoskeleton.

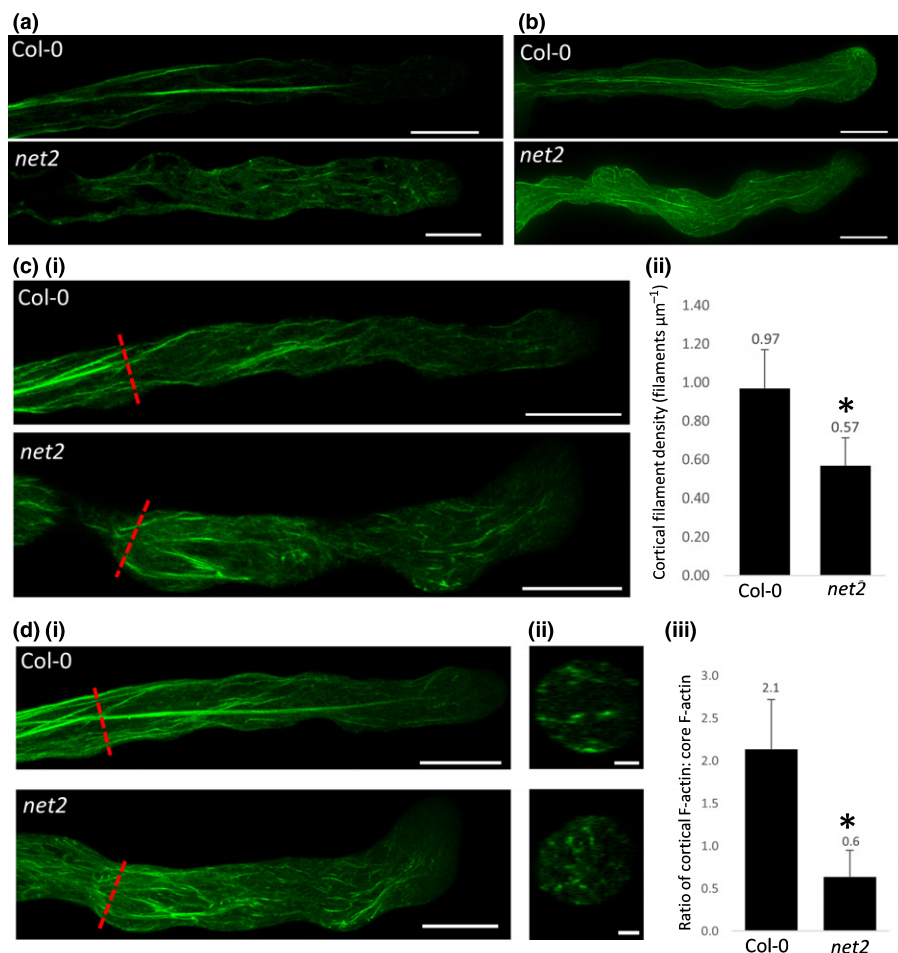
## Polar transport and positioning of organelles is impaired in *net2* mutant pollen tubes

The actin cytoskeleton regulates cytoplasmic streaming, organelle positioning and tip-focussed transport of secretory vesicles (SVs) in growing pollen tubes (Vidali & Helper, 2000). To better understand how NET2 proteins may regulate actin-driven pollen tube growth, we proceeded to investigate whether NET2 proteins have important functions in regulating tip-focussed vesicle transport and cytoplasmic streaming.

To examine tip-focussed vesicle transport in growing pollen tubes, we analysed the subcellular localisation of SVs in pollen tubes stably expressing the secretory vesicle marker GFP-RabA4B (Zhang *et al.*, 2010) under the *pLAT52* promoter. The localisation pattern of GFP-RabA4B in *net2a/net2b/net2c/net2d* mutant pollen tubes was observed to be markedly different from that of Col-0 (Fig. 6a-i). Whilst Col-0 pollen tubes exhibited a classical inverted cone-shaped localisation pattern at the pollen tube tip (Zhang *et al.*, 2010), the *net2* quadruple mutant pollen tubes displayed a massive tip-focussed accumulation of GFP-RabA4B and loss of the ordered inverted cone distribution pattern. Col-0 pollen tubes were noted to possess a 2.7-fold ratio of GFP-RabA4B-labelled SVs at the pollen tube tip compared with the distal shank region of the cell. In comparison, the tip:distal ratio of SVs at the tip of mutant pollen tubes was 6.9, constituting a statistically significant increase ( $P < 0.01$ ). The data indicate a role for NET2 proteins in the regulation of tip-focussed vesicle transport.

A role for NET2 proteins in the regulation of cytoplasmic streaming was also investigated. Visualisation of cytoplasmic streaming was performed by monitoring the transport of mitochondria in pollen tubes labelled by Mitotracker™ Red. We observed defects in transport and positioning of mitochondria in *net2a/net2b/net2c/net2d* mutant pollen tubes (Fig. 6b; Video S2). Whilst Col-0 pollen tubes exhibit rapid anterograde and retrograde transport of mitochondria, the longitudinal movement of mitochondria in *net2* quadruple mutant pollen tubes was perturbed, with reduced mitochondrial velocity and frequent short circular trajectories clearly observable. Furthermore, in *net2* quadruple mutants, abnormal accumulations of mitochondria in the pollen tube subapex was also evident, but were not observed in Col-0 tubes. It can therefore be concluded that NET2 proteins are important in the organisation of cytoplasmic streaming and organelle positioning.

Taken together, our data indicate an association of NET2 proteins with F-actin during pollen tube development and pollen tube growth, leading us to speculate a role for NET2 in the regulation of the pollen cytoskeleton. We demonstrated that NET2 proteins are necessary for normal pollen tube growth, organisation of the pollen tube cytoskeleton and actin-dependent subcellular processes such as polar vesicle trafficking and cytoplasmic streaming.

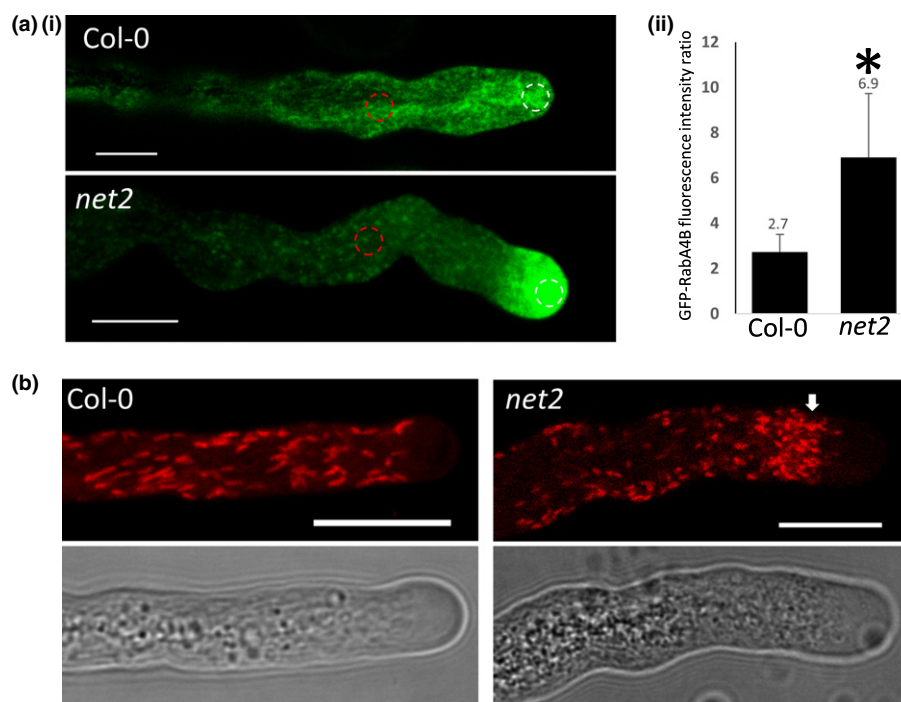


**Fig. 5** The actin cytoskeleton is disorganised in Arabidopsis *net2a/net2b/net2c/net2d* mutant pollen tubes. The actin cytoskeleton was analysed in Col-0 and *net2a/net2b/net2c/net2d* mutant pollen tubes expressing the actin marker, Lifeact-mNeonGreen. (a) Single-plane confocal cross-section of Col-0 and mutant pollen tubes expressing Lifeact-mNeonGreen. (b) 3D Z-projection of the actin cytoskeleton of Col-0 and mutant pollen tubes. (c) Density of F-actin filaments at the cell cortex (number of filaments/ $\mu\text{m}$ ) is reduced in mutant pollen tubes. (i) Single-plane confocal image of Lifeact-mNeonGreen at the cell cortex of Col-0 and mutant pollen tubes. The density of cortical actin filaments was calculated 50  $\mu\text{m}$  distal to the pollen tube tip (indicated by the red line). (ii) The density of F-actin filaments at the cell cortex was significantly reduced in the mutant. (10 Col-0 and 10 mutant pollen tubes were analysed). (d) The proportion of cortical actin filaments relative to core actin filaments is reduced in mutant pollen tubes. (i) 3D Z-projection of Col-0 and mutant pollen tubes expressing Lifeact-mNeonGreen. Analysis was performed 50  $\mu\text{m}$  distal to the pollen tube tip (indicated by the red line). (ii) Orthogonal cross-sections of Col-0 and mutant pollen tubes shown in 5(d-i) at the position indicated by the red line. (iii) The ratio of cortical F-actin filaments (within 1  $\mu\text{m}$  of the plasma membrane) relative to core actin filaments ( $>1$   $\mu\text{m}$  from the plasma membrane) was greatly reduced in mutant pollen tubes. Analysis was performed on 10 Col-0 and 10 mutant pollen tubes, 50  $\mu\text{m}$  distal to the pollen tube tip. The asterisk indicates statistical significance ( $P < 0.01$ ). Bars: 10  $\mu\text{m}$  (a, b, c, d-i); 2  $\mu\text{m}$  (d-ii). Chart error bars represent standard deviation of the mean.

## Discussion

The actin cytoskeleton has an essential role in pollen development, germination and pollen tube growth (Gervais *et al.*, 1994; Gibbon *et al.*, 1999; Zonia *et al.*, 1999; Vidali *et al.*, 2001). Our data suggest an important role for NET2 proteins in angiosperm fertilisation and the regulation of the pollen cytoskeleton during gametogenesis, pollen germination and tube growth. The observation of dynamic NET2A localisation to the actin cytoskeleton during pollen development and tube growth suggests its involvement in the regulation of actin-driven processes occurring during the Arabidopsis male reproductive cycle, whilst phenotypic defects observed in *net2* mutant pollen confirm the crucial role for NET2 in the normal regulation of actin-driven pollen tube growth.

The actin cytoskeleton has not yet been characterised during the course of pollen grain development and our data provide a novel illustration of the dynamic structure of the actin cytoskeleton in live developing Arabidopsis pollen grains, in previously unobserved detail. Our data demonstrate that NET2A-GFP reorganises to unique structures at specific stages of microgametogenesis; localising to the actin cytoskeleton over the course of pollen grain development. We first observed NET2A-GFP in bicellular pollen as punctae distributed throughout the cytoplasm, differing from previous observations of plasma membrane-localised NET2A in pollen tubes (Duckney *et al.*, 2017). This is possibly because in growing pollen tubes, PRK4 and PRK5 recruit NET2A to the plasma membrane (Duckney *et al.*, 2017), but are not abundantly expressed in early pollen development (Winter



**Fig. 6** Polar transport of organelles is disrupted in Arabidopsis *net2a/net2b/net2c/net2d* mutant pollen tubes. (a) Apical accumulation of secretory vesicles in the *net2a/net2b/net2c/net2d* mutant. (i) Secretory vesicles labelled by GFP-RabA4B in Col-0 and *net2a/net2b/net2c/net2d* mutant pollen tubes. (ii) Apical vesicle accumulation was calculated as apical GFP-RabA4B fluorescence ratio (white apical region of interest (ROI):red distal ROI, 30  $\mu$ m distal to tip). Analysis was performed on eight Col-0 and eight mutant pollen tubes. The asterisk indicates statistical significance ( $P < 0.01$ ; as calculated using Student's *t*-test). Chart error bars represent standard deviation of the mean. (b) Subapical accumulation of mitochondria in the *net2a/net2b/net2c/net2d* mutant, indicated by the white arrow. Mitochondria of Col-0 and mutant pollen tubes were stained using Mitotracker red. Bars, 10  $\mu$ m.

*et al.*, 2007). Later in bicellular pollen development, NET2A was observed to localise to F-actin in a so-called 'transverse array', in which a polarised, transversely aligned belt of actin cables was observed at the cell cortex. Following this, in late-bicellular and tricellular pollen NET2A-GFP localised to a filamentous actin array, connected to the nuclear envelope of the VCN, with filamentous networks of actin extending to a network of cortical F-actin filaments. In mature tricellular grains, NET2A-GFP decorated a cortical array of actin filaments, consisting of a dense meshwork of fine filaments at the cell cortex. Our data drew significant similarities to, and extends, previous studies conducted on the actin cytoskeleton of fixed *in vitro*-cultured pollen grains from *N. tabacum* and *B. napus*. It is therefore, possible to infer the potential functions of each actin structure and consequently those with which NET2A may be involved. Similar actin structures have been hypothesised to regulate pollen cell elongation, nuclear positioning and restructuring of the plasma membrane (Gervais *et al.*, 1994; Zonia *et al.*, 1999; Kang *et al.*, 2003; Yamamoto *et al.*, 2003) and it is possible that NET2 proteins may contribute to the robustness of pollen grain developmental processes through an association with F-actin. Notably, NET2A-GFP was observed to bind to actin at the cell cortex of developing pollen grains, where it may serve to establish AMCSs, as reported in growing pollen tubes (Duckney *et al.*, 2017) and was also observed to localise to F-actin at the VCN. The actin cytoskeleton is known to be physically tethered to the outer nuclear envelope in Arabidopsis (Tamura *et al.*, 2013; Zhou *et al.*, 2014).

As such, it is possible that NET2 proteins may mediate connections between the actin cytoskeleton and nuclear envelope in Arabidopsis pollen grains.

During germination, NET2A was observed to be recruited from the cytosol to membrane-localised foci, specifically at the site of pollen tube outgrowth. Following pollen germination, these highly localised NET2A punctae at the VC cortex are continuous with the NET2A punctae of previously characterised AMCSs at the pollen tube shank (Deeks *et al.*, 2012; Duckney *et al.*, 2017). This led us to propose that the NET2A punctae observed in germinating pollen were also AMCSs. As the growing tube extends, cytosolic NET2A is likely to be recruited to membrane foci to establish AMCSs as the membrane becomes increasingly distal to the growing tip and gains shank identity. Actin has a principle role in pollen germination (Gibbon *et al.*, 1999), during which F-actin cables reorganise to align longitudinally to the germination site, protruding into the nascent tube and later forming the longitudinal actin cables of the pollen tube shank (Volger *et al.*, 2015). It is tempting to speculate that NET2A may bind actin to the plasma membrane at the site of germination, perhaps to support targeted pollen tube outgrowth and later organise longitudinal actin cables at the cell cortex of the shank as the pollen tube extends.

Our investigation of *net2* loss-of-function mutants clearly confirmed an important role for NET2 proteins in the male gametophyte and we have noted that each member of the NET2 protein family subclade functions in the regulation of pollen tube growth.



Interestingly, NET2 did not appear to be necessary for pollen grain development as we could not identify any major defect in actin organisation in the *net2* quadruple mutant, perhaps due to functional redundancy with other NETs known to be expressed in pollen grains, such as NET3 (Wang *et al.*, 2014). The ‘zig-zag’ morphology phenotype of mutant pollen tubes was determined to result from a loss of ability of mutant tubes to maintain a constant growth trajectory. Pollen tube growth trajectory is maintained by homeostatic signalling cascades, modulated in response to extracellular signals (Guan *et al.*, 2013; Takeuchi & Higashiyama, 2016; Schleible & McCubbin, 2019). We have recently demonstrated that NET2 interacts with PRKs, known regulators of actin-driven pollen tube growth and targeting in response to extracellular signals (Takeuchi and Higashiyama, 2016). From our data, it could be speculated that NET2 may play a role in signalling to the actin cytoskeleton downstream of PRKs to maintain normal pollen tube growth trajectory. In support of this hypothesis the phenotypic growth defects of *net2* pollen appeared more pronounced during growth through the stigma, indicating a potential role in extracellular signal transduction to the cytoskeleton in the regulation of actin-driven growth. The observed ‘zig-zag’ phenotype is a curious one and, to our knowledge, mutations of no other cytoskeletal-regulatory proteins result in a similar pollen tube defect. Our data therefore demonstrate a unique and crucial role for NET2 proteins in normal pollen tube growth, perhaps in signal transduction during fertilisation.

The defective pollen tube growth phenotype can be attributed to loss of AMCSs at the pollen tube plasma membrane. Consistent with the hypothesised role for NET2 in anchoring actin to the plasma membrane (Duckney *et al.*, 2017), we observed a reduction of cortical F-actin in *net2* quadruple mutant pollen tubes that is likely to represent unpinning of actin cables from the membrane. The actin cytoskeleton was disorganised, misaligned and fragmented in mutant pollen tubes, indicating a role for NET2 at AMCSs in structural organisation of the pollen tube cytoskeleton, which may serve as points of stabilisation and structural orientation of longitudinal actin cables. The importance of NET2 in the regulation of the pollen tube cytoskeleton was further evidenced in defects in actin-driven subcellular processes including cytoplasmic streaming and vesicle transport. Longitudinal actin cables are important in rapid, long-distance transport of organelles (Vidali *et al.*, 2001), so it is therefore unsurprising that mitochondrial movement was impaired and with loss of defined trajectory in mutant pollen. Taken together our data indicate that *net2* loss of function indirectly affects mitochondrial movement as a result of impairment of the actin cytoskeleton. Disruption to cytoplasmic organisation was also evident in mutant tubes, with abnormal accumulations of mitochondria in the pollen tube subapex and aberrant distribution patterns of SVs at the tip. We propose that impairment of actin-driven streaming in mutant pollen tubes prevents active mixing of organelles leading to a ‘phase separation’ small SVs at the extreme apex and shank-restricted large organelles. Pollen tube growth occurs only at the tip through targeted apical secretion. The abnormal

transport of SVs in mutant pollen tubes is likely to have been causative of the defective pollen tube growth here observed.

Taken together, we demonstrate dynamic localisation of NET2 to the actin cytoskeleton throughout each stage of pollen development, pollen germination and pollen tube growth, indicative of an involvement of NET2 in the organisation of actin in the male gametophyte. In particular, we demonstrate an essential role for NET2 proteins in normal pollen tube growth, with functions in the organisation of F-actin and organelle transport.





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## Author contributions

PJH conceived the project, which was supervised by MJD and PJH. Most of the experiments were performed by PD, with exception of the generation of the *NET2C* CRISPR constructs and Lifeact-mNeonGreen construct, which was performed by JK. Analysis of developing pollen grains was performed by both PD and MRD. PD prepared the figures and wrote the manuscript with MJD, TJH and PJH.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Pollen tube morphology defects are present in specific *net2* loss-of-function mutant combinations.

**Fig. S2** Pollen tube growth of *net2* mutants is reduced in semi-*in vivo* germination assays.

**Fig. S3** Aberrant pollen tube growth of *net2* mutants through the stigma during fertilisation.

**Fig. S4** The actin cytoskeleton of *net2* mutant pollen grains is not defective.

**Table S1** Primers used in this study.

**Video S1** *net2* mutant pollen tubes are unable to maintain a constant growth trajectory.

**Video S2** Cytoplasmic streaming in Col-0 and *net2* mutant pollen tubes stained with Mitotracker Red.

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